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**Title Page**

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**Abstract**

**Objectives**

The objective of this study was to explore potential relationships between neonatal line (NNL) width and early life history variables such as maternal health, gestation, the birth process, and perinatal health.

**Materials and Methods**

Histological thin sections of deciduous canines were studied from 71 children from the Avon Longitudinal Study of Parents and Children (ALSPAC). The width of the NNL was measured in three locations on the tooth crown using spatial mapping techniques (ArcGIS) from digital images from an Olympus VS-120 microscope. Life history variables were collected prospectively through a combination of clinical observations and questionnaires.

**Results**

Infants born late term or post term had narrower neonatal lines than those born prematurely or at full term. Infants born in Autumn (September to November) had narrower NNLs than those born at other times of year. NNLs in infants born to mothers with hypertension were wider than those without. Infants resuscitated at birth or born to obese mothers had narrower NNLs than those that were not. There was no association between NNL width and either the type or duration of delivery.

**Discussion**

The NNL in enamel is an irregular accentuated line, but the factors underlying its formation and width remain unclear. In contrast to some previous studies, we found no association between wider NNLs and long or difficult births. Instead we found that the width of the neonatal line NNL varied in relation to parameters that reflected the prenatal environment and length of gestation.

**Life history and dental enamel**

Early life history can be investigated using light microscopy of histological ground sections of deciduous teeth using evidence from the regular structure of enamel growth (Birch and Dean, 2009, 2014; Nava *et al.*, 2017). Disruptions to the regular incremental pattern of enamel formation can be used to identify and reconstruct a sequential chronology of events that affected the growth and development of an individual in the past (Hillson, 1992). Daily incremental markings in enamel are visible in transmitted polarized light microscopy (PLM) (Dean, 1987; Boyde, 1989; Shellis, 1998). These are formed during enamel matrix secretion and do not remodel thereafter. In this way the microstructure of dental enamel preserves a chronological record of growth that enables the identification of both regular growth increments and those that have been superimposed upon this as irregularly spaced structural features of varying width and intensity when viewed in PLM (FitzGerald, 1998; Antoine *et al.*, 2009).

Enamel is comprised of prisms, or rods, that are made up of tightly packed bundles of enamel crystallites (Boyde, 1964; Boyde, 1989). Prisms run from the enamel dentine junction (EDJ) to the enamel surface. Groups of prisms follow the path of ameloblasts, the cells that secrete enamel matrix, that creates a template for subsequent crystallite and prism mineralization (Robinson *et al.*, 1997). At regular intervals along the length of every prism fine dark markings, known as cross-striations, denote daily increments of enamel formation (Boyde, 1989; Antoine *et al.*, 2009). Superimposed upon this daily incremental pattern is a regular longer period incremental marking, with a periodicity of between six to twelve days in permanent human teeth (FitzGerald, 1998; Reid and Dean, 2000; 2006) and between five and

nine days in human deciduous teeth (Mahoney, 2011, 2012). In studies of modern human permanent teeth, the modal value appears consistently to be eight days (Smith et al 2007). These regular long-period markings or striae (of Retzius) are coarser incremental markings that run obliquely through the enamel from the EDJ to the enamel surface, where they emerge in the grooves between ridges on the enamel surface known as perikymata. Both regular long-period striae (of Retzius) and perikymata grooves are less pronounced in deciduous than in permanent enamel (Hillson, 2014). **Figure 1** illustrates the relationship between these various enamel incremental markings.

Interruptions to these regular growth increments, in the form of altered rate of enamel formation, are considered to be indications of disruption to the individual's normal developmental process (Boyde, 1989; Boyde, 1990). Interruptions to growth are often visible as external structural defects (enamel hypoplasia, or EH) and/or as internal structural defects that represent temporal disruptions to the enamel-forming ameloblast cell sheet (Hillson and Bond, 1997). The internal structural defects are manifest in PLM as irregular accentuated striae (or IAS) illustrated in **Figure 1**. These accentuated markings or striae (IAS) may be caused by disruption of or change of rate in enamel matrix secretion. Separately, or in addition to this, there may be changes in the quality or orientation of mineral laid down during the secretory phase. IAS may appear as either darker or broader lines or bands in PLM and represent the position of the ameloblast cell sheet at the time of the disruption. IAS may either coincide with the regular long period striae (of Retzius) or be superimposed between them in an irregular manner (Hillson, 2014). They may or may not reach the enamel surface and present as EH (Hillson and Bond, 1997; Witzel *et al.*, 2008).

There is little consensus as to what physiological stress events or pathologies underlie the appearance of IAS of different widths, intensities (dark or bright) or of different lengths of expression (short or long) within enamel (Boyde, 1990; Kierdorf and Kierdorf, 1997; Witzel *et al.*, 2008). The relationship between external and internal markers of enamel growth disruption (IAS and EH) is incompletely understood (Witzel at al 2008, Hillson 2014) and our incomplete understanding of the relationship between life experience and changes to the microstructure of enamel remains a major stumbling block in the interpretation of life history from dental histology.

A number of pathologies and stress events are known to disrupt enamel formation. These include genetic or inherited conditions such as amelogenesis imperfecta (Garn *et al.*, 1965; Pindborg, 1982; Bhat and Nelson, 1989; Witkop, 1989; Flanagan *et al.*, 1997; Alt and Türp, 1998; Klingberg *et al.*, 2002); maternal disturbances including maternal diabetes, hypothyroidism and hypertension in pregnancy (Gregg, 1944; Kreshover *et al.*, 1954; Grahnen and Edlund, 1967; Guggenheimer *et al.*, 1971; Norén, 1984; Silva-Sousa *et al.*, 2003; Dolphin and Goodman, 2009; Vucic *et al.*, 2017); variables reflecting prenatal experience such as gestational age, very low birth weight, and perinatal Vitamin Deficiency (Grahnen and Larsson, 1958; Norén, 1983; Seow *et al.*, 1984a; Seow *et al.*, 1984b; Pimlott *et al.*, 1985; Seow, 1986; Fearne *et al.*, 1990; Franco *et al.*, 2007; Rythen *et al.*, 2008; Priya *et al.*, 2015); or subsequent conditions associated with birth and early neonatal life including trauma such as intubation (Schour and Kronfeld, 1938; Johnsen *et al.*, 1984; Eli *et al.*, 1989; de Oliveira Melo *et al.*, 2014) as well as stress events or systemic disturbances to health occurring while the deciduous dentition is being formed. In many populations EH and IAS in early forming deciduous teeth are likely to result from the combined effect of several

conditions such as malnutrition, parasitic load, and infectious diseases, and fevers (Hillson, 2014).

### *The neonatal line*

The neonatal line (NNL) has been firmly established as a specific type of accentuated line that represents a histologically observable disturbance in enamel microstructure formed at or around the time of birth (Schour, 1936; Stein, 1936). The work of Rushton (1933), following that of Rygge (1916) and von Ebner (1903), identified the NNL as a linear feature that appears brown in transmitted light microscopy (TLM) and blue in reflected light (Boyde, 1964; Boyde, 1989; Hillson, 2014) and which obliquely transects the long axis of the enamel crown. It occurs in teeth that are forming at the time of birth (all deciduous teeth in most cases the first permanent molars). The position of the NNL within the enamel differs from tooth type to tooth type, as does the quality of the enamel on either side of the NNL (Schour, 1936). The NNL has been observed in almost all (90-100%) longitudinal histological sections of teeth that are forming at birth from live-born children (Schour, 1936; Massler *et al.*, 1941; Sarnat and Schour, 1941; Gustafson and Gustafson, 1967; Weber and Eisenmann, 1971; Norén *et al.*, 1978a; Whittaker and Richards, 1978; Eli *et al.*, 1989; Skinner and Dupras, 1993; Sabel, 2012; Canturk *et al.*, 2014; Hurnanen *et al.*, 2017).

### Factors hypothesized to be associated with neonatal line appearance

While the NNL can be securely identified and associated with birth by its relative position within the enamel crown (Skinner and Dupras, 1993), there is still considerable variation in it's appearance that remains unexplained. In particular, the width or breadth of the NNL varies from 3 to about 30 μm (Weber and Eisenmann, 1971; Eli *et al.*, 1989; Zanolli *et al.*, 2011; Sabel, 2012; Hurnanen *et al.*, 2017) when viewed in PLM in longitudinal histological

ground sections of deciduous teeth (as seen in **Fig. 2**). As with other accentuated histological enamel markings, a variety of etiologies have been proposed to explain this variation, each having a specific implication for the processes by which enamel microstructure records life history experiences. Factors theorized to affect the appearance of the NNL fall largely into two categories a) maternal and gestational variables, pertaining to maternal health and the fetal environment; or b) aspects of the birth process and early neonatal life variables affecting the child at or soon after birth.

NNL width can be measured in a number of ways and non-uniformity in the way it has been measured likely contributes to the variation reported in the literature. The majority of studies have emphasized the importance of measuring width across the NNL along the prism path to capture the duration of NNL formation, but there are exceptions (Sabel *et al.*, 2008). A further consideration is the potential impact of section obliquity. A plane of section that cuts through the NNL at an oblique angle to the true long axis of the tooth that runs through the cusp tip and dentine horn and pulp horn tip will elongate and thicken the appearance of the NNL. Only a few studies have reported in detail exactly where along the NNL width measurements were taken (Zanolli *et al.*, 2011). In some cases measures of NNL widths have even been pooled between several tooth types (Eli *et al.*, 1989; Hurnanen *et al.*, 2017). Given that there is variation in the daily enamel formation rate, which increases from the EDJ to the enamel surface and differs across tooth types (Mahoney, 2012; Birch and Dean, 2014), the width of the NNL along a prism path crossing a NNL at any given point will vary with both tooth type and its location within the enamel crown.

***Maternal and gestational variables hypothesized to be associated with NNL width***

The biological status of the mother and the resulting fetal environment are potential factors in determining NNL width. Several studies have concluded that the appearance of the NNL is primarily dependent on gestational factors (Norén *et al.*, 1978a; 1978b; Norén, 1984; Zanolli *et al.*, 2011; Kurek *et al.*, 2015; Behie and Miszkiewicz, 2019). Norén and colleagues reported that wider NNLs are related to disruptions in the normal fetal calcium-phosphate metabolism caused by gestational illnesses such as maternal diabetes (Norén *et al.*, 1978a; Norén, 1984). Subsequent research recorded a thinner NNL associated with conditions in pregnancy requiring an antispasmodic medication (Kurek *et al.*, 2015). Others have concluded that lower gestational age at birth or lower birthweight co-occur with wider NNLs (Norén, 1983; Rythen *et al.*, 2008; Zanolli *et al.*, 2011; Kurek *et al.*, 2015; Priya *et al.*, 2015). Moreover, recent studies have reported an association between season of birth and both the width of the NNL (Kurek *et al.*, 2015) and prenatal enamel thickness (Żądzińska *et al.*, 2013). Children born in the summer and in the spring had thinner NNLs than children born in the winter. The authors suggested that lower maternal and fetal Vitamin D exposure in winter births contributed to a delay in the resumption of regular secretion by ameloblasts (Kurek et al 2015). Most recently, Behie and Miszkiewicz (2019) reported that children born to mothers who consumed alcohol during pregnancy had a thicker NNL than those of mother who abstained.

***Birth processes and early neonatal life variables hypothesized to be associated with NNL width.***

There is a large clinical literature that attributes variation in the width of the NNL to factors that reflect the severity of the disruption to infant homeostasis caused by delivery, including the difficulty (mode or duration) of the birth process and measures of infant health immediately after birth. The ‘homeostasis’ model places more emphasis on short-term

disruptions to health, rather than longer-term underlying metabolic aspects, as factors influencing the width of the NNL.

Early studies attributed the presence of the NNL to the abrupt change in environment and nutrition experienced at birth and speculated that the prominence of the NNL should be proportionate to the difficulties and disturbances experienced at birth and in the early postnatal period (Schour, 1936; Stein, 1936; Schour and Kronfeld, 1938). Subsequently, Massler *et al*. (1941) emphasized factors including neonatal distress and clinical intervention around the time of birth. Following on from this, several studies have evaluated the association between NNL width, as a measure of stress, and mode of delivery. In several studies vaginal delivery was associated with wider NNLs compared to delivery by Caesarean section and this difference has been attributed to the longer duration of systemic stress associated with the natural birth process (Eli *et al.*, 1989; Canturk *et al.*, 2014). Conversely, other studies have reported that there was no variation in the width of the NNL according to mode of delivery (Zanolli *et al.*, 2011; Kurek *et al.*, 2015; Hurnanen *et al.*, 2017). A potential confounding factor unaddressed by these studies is that some natural births are relatively straightforward while some Caesarian deliveries are non-elective and can occur after many hours of labor and physiological stress to both mother and baby (Kurek et al 2015). One recent study unexpectedly found that prolonged duration of vaginal delivery was associated with a narrower NNL (Hurnanen *et al.*, 2017)

**Objectives of this study**

This paper examines the factors associated with NNL width in one specific tooth class, the deciduous canine, by evaluating the width of the NNL in relation to a series of variables reflecting gestation, birth timing and condition and the circumstances surrounding delivery.

Using comprehensive prospectively collected data this study tested the associations between NNL width and prenatal and gestational variables, and between NNL width and the circumstances of birth. This study also examined NNL width across different parts of the crown. Finally, it compares NNL width in teeth eight individuals in which a single child contributed more than one deciduous canine in order to illuminate variation within the same individual.

## MATERIALS AND METHODS

### Materials

Deciduous canine teeth from 71 children enrolled in the Avon Longitudinal Study of Parents and Children (ALSPAC) were included in this analysis. ALSPAC is a prospective observational cohort study that investigates influences on health and development across the life course (Boyd *et al.*, 2013; Fraser *et al.*, 2013). The study enrolled 14,541 pregnant women resident in South West England with expected dates of delivery between 1st April 1991 and 31st December 1992. Deciduous teeth were collected from ALSPAC participants between 1997 and 2004. Families were asked to send the study up to four naturally shed teeth per child as soon as possible after loss. Teeth were stored at -20$^{o}$C until released for analysis. Information on maternal and infant health and environmental exposures were obtained from self- reported questionnaires and from routinely collected clinical data recorded during pregnancy and shortly after birth were extracted from the medical notes. More detailed obstetric information including records of medical interventions, mode of delivery, duration of labor, and vital statistics of mother and baby were obtained for a subset of ALSPAC participants, including 32 individuals studied here. Ethical approval for the study was

obtained from the ALSPAC Ethics and Law Committee and the Local Research Ethics Committees.

One tooth from each child was used for the main part of the study. Eight of the children were represented by more than one tooth (making a total of 80 teeth in the sample); however, the nine additional 'matching' teeth are excluded from all analyses except those comparing NNL width across teeth from the same individual. Deciduous canines were preferentially selected as part of the sampling regime for a more comprehensive ongoing study of enamel composition and structure because of their relatively low levels of cuspal wear, greater enamel thickness, and longer enamel formation time. The NNL in the canine tooth is entirely contained in the cuspal enamel and it may be least affected by short-term cessation of ameloblast function at birth (Hurnanen *et al.*, 2019).

**Methods**

The NNL was identified in histological thin sections taken through the center of the enamel cusp of each canine, approximately 300 μm from the exact midline running between cusp and cervix of each tooth. These were cut, lapped and polished using standard methods described below. Unlike deciduous incisors, that have an incisal edge, deciduous canines have a pointed dentine horn. However, avoiding obliquity is difficult and ideally an axial longitudinal section through a deciduous canine tooth must pass exactly through the cusp tip, the dentine horn and the pulp horn (see discussion in Birch 2012). A low speed *Buehler Isomet* circular diamond saw was used to section the teeth from the midline. These sections were mounted on glass slides with Araldite *Huntsman 2020* and then hand-lapped with successively fine

carborundum grit papers (600, 1200) to a thickness of approximately 100 μm and polished with aluminum oxide powder suspended in water. Coverslips were mounted temporarily with glycerine for polarized light microscopy. The slides were then imaged using an Olympus VS-120 virtual slide scanning system at resolutions of 2x, 20x, and 40x magnification under polarized light. The slide scanning system created very high-resolution digital images that were then imported with spatial coordinates intact into a geographic information system (ArcGIS, ESRI USA).

The width of the NNL is defined as the length of a prism spanning the start and finish of the NNL. The boundaries of the start and finish of the NNL were identified at high magnification (20x objective) as either a marked or gradual change in enamel color intensity in transmitted PLM (**Figure 2**). In all cases the width of the NNL was measured along the path of prisms running through the line multiple times and in three locations along the line: cuspally, in the middle third, and cervically (nearer the enamel-dentine junction, or EDJ) and their location and length digitally recorded in ArcGIS. Using multiple measures takes into account variability in daily enamel secretion rates across the crown (Birch and Dean, 2009) and offers an averaged measure of NNL thickness, following the measures presented by Zanolli *et al*. (2011). One of the advantages of using repeated measurements in three distinct regions of the NNL is that individual observer variability may be reduced through the averaging process. Measurement location is important, since previously Sabel *et al* (2008) have reported that NNL widths are highest in the middle third of the tooth crown. Since enamel daily secretion rates are higher in the cuspal enamel than in enamel close to the EDJ in the position where the NNL is observed (Birch and Dean, 2009, 2014), we expected NNL width to be highest in cuspal enamel and lowest in the most cervical enamel of the tooth crown where the NNL is close to the EDJ.

Data on mothers and infants included in the ALSPAC longitudinal cohort study sample were collected for the purpose of describing details of maternal health and lifestyle, pregnancy, and birth; and then details of the subsequent growth, development and health of the child. The study website contains details of all the data available through a fully searchable data dictionary and variable search tool (http://www.bristol.ac.uk/alspac/researchers/our-data/): relating to pregnancy, delivery, and the first few weeks of life as well as questionnaires completed by the mother in the same period. Informed consent for the use of data collected via questionnaires and clinics was obtained from participants following the recommendations of the ALSPAC Ethics and Law Committee at the time. In addition, in-depth information abstracted from clinical obstetric records of delivery were available for a subset of 32 of the 71 infants included in this study. Variables encompassing maternal and gestational factors as well as those characterizing the birth process and early neonatal life factors were selected.

Maternal or gestational factors selected include: maternal height, weight (Pre-pregnancy) BMI (Pre-pregnancy), obesity, age (with advanced maternal age as over 35 years), parity; fetal sex, birth weight, crown-to-heel length, gestational age at birth, status as small for gestational age; maternal Vitamin D levels during pregnancy and the occurrence of maternal diabetes, preeclampsia or hypertension, season of birth; also any responses to questionnaires given at clinic visits at 32 weeks gestation reporting maternal infection, maternal injury or shock, or maternal vomiting during the three months prior, so between 20-32 weeks, and any maternal infection, maternal injury or shock from seven months until the end of pregnancy reported in the eight week questionnaire. Gestational metabolism variables included gestational diabetes, gestational hypertension, glycosuria, preeclampsia, maternal Vitamin D levels, and cord blood calcium. These were examined alongside descriptors of pregnancy

outcome (season of birth, gestational age, birthweight, crown-heel height). Evidence of significant health disruption to the mother during pregnancy (injury, vomiting, infection) was also considered. For this study, season of birth was derived from the child's age and date of completion of a questionnaire after birth.

Gestational age as reported in this study follows the American College of Obstetricians and Gynecologists standard with 'Preterm' as birth prior to 37 weeks gestation; 'Early Term', 37 to 38 weeks; 'Full Term', 39 to 40 weeks; 'Late term', 41 weeks; and 'Post Term', 42 weeks and beyond. Status as small for gestational age is based on birthweights in the $10^{th}$ percentile for each gestational week as presented by Talge and colleagues (2014). Maternal BMI was calculated according to Keys *et al.* (2014) and obesity defined as a BMI of 30kg/$m^2$ or higher.

Delivery variables were recorded at birth unless otherwise specified. These include mode of delivery: Caesarean birth, spontaneous vaginal birth , or instrument assisted birth (assisted breech birth, or use of forceps or vacuum). Births were categorized as 'spontaneous' vs. those requiring 'intervention' by combining c-section, instrument assisted, and vacuum assisted births as interventions against spontaneous, or non-instrument-assisted, vaginal birth. Duration of first stage of labor (hours), duration of second stage of labor (minutes), total duration of labor (minutes), and maternal distress were also assessed from data reported to ALSPAC. Neonate health variables assessed included abnormal fetal heart rate, APGAR score at one minute after birth (a standard measure of neonate health; Apgar, 1953; Apgar *et al.*, 1958), APGAR score at five minutes after birth, and whether the baby was resuscitated. Early life health (in the first 14 days of life) was assessed based on reported occurrence of jaundice, feeding problems, or pyrexia (fever).

Statistical relationships between the NNL widths and the study variables are described using linear models (function lm) as implemented in the R base package (R Core Team, 2018). The $\omega^2$ value is calculated using the anova_stats function implemented in R package sjstats (Lüdecke, 2019), and the measure of effect size, Cohen's D, is calculated using cohen.d from the effsize package (Torchiano, 2014).

## RESULTS

Average NNL widths in the three tooth crown locations vary from 2.06 to 30.13 μm and fall within the range reported by previous studies (Eli *et al.*, 1989; Zanolli *et al.*, 2011; Sabel, 2012; Hurnanen *et al.*, 2017). The relative width of the NNL compared at three different locations within the tooth crown across the sample was found to vary greatly. The distribution largely follows a pattern expected from the daily enamel formation rate in each region of the crown (**Figure 3, Table 1**), with the largest widths measured in the coronal (cusp) third of the line, decreasing through the middle of the line to the smallest widths in the cervical third of the line. Mean NNL widths in the three tooth crown regions varied greatly but were highly correlated across the sample (r = 0.79 for the middle and the cusp; r = 0.76 for the middle and EDJ; and r = 0.76 for the cusp and EDJ). Associations between variables tested and the width of the NNL observed in all three regions of the tooth crown are given in **Table 2**.

### *Maternal Variables*

Descriptors of maternal status in terms of age, weight, BMI, obesity, parity, and history of hypertension or diabetes prior to pregnancy were examined (**Table 3**). Maternal age, height, and parity had no effect on NNL width. Maternal obesity (a pre-pregnancy BMI of over 30, though n = <5) had medium to large effect on decreasing the width of NNL line. A history of

hypertension (n = 10) was correlated with an increase in NNL width, with medium to large effect sizes.

***Gestational Variables***

Descriptors of gestational variables are given across three broad categories (**Table 4**).

***Maternal Metabolic Disruption***

A small number of mothers (n = 7) developed hypertension during pregnancy; this condition was associated with increased width of the middle portion of the NNL in their children. None of the mothers in the sample developed pre-eclampsia or maternal diabetes, and three developed glycosuria. Maternal infection, injury or shock, and other health disruptions during pregnancy (as specified in Table 4) were not associated with increased NNL width. Experience of health disruption in pregnancy (either infection, injury, or vomiting) was not associated with changes in NNL appearance.

Previous research has demonstrated the potential for interaction between Vitamin D metabolism (including calcium levels), birth weight, gestational age, and seasonality of birth (Doi *et al.*, 2011; Day *et al.*, 2015), with possible implications for enamel development (Norén *et al.*, 1978a; Żądzińska *et al.*, 2013; Kurek *et al.*, 2015). Cord blood calcium was measured in a small number of cases (n = 13) but was not associated with NNL width. Maternal Vitamin D, however, has a slightly more complicated set of interactions.

Maternal serum total 25-hydroxy Vitamin D (25[OH]D) levels vary throughout the year, in relation to exposure to sunlight, as demonstrated previously in this sample (Sayers *et al.*, 2009). Because maternal Vitamin D was sampled at different gestational stages, at different

times of the year, it is necessary to detrend the series to account for the clear pattern of seasonal increase and decrease (**Figure 4**). Utilizing a detrended measure (the observed amount of 25H[OH]D divided by the average for the month observed), increased maternal Vitamin D levels were related to decreased NNL width, though only strongly in the cusp ($p = 0.0122$, $r^2 = 0.1389$). While a linear model using both the level of Vitamin D and the month of testing did not show a significant correlation to the width of the NNL in the three regions of the tooth, levels sampled in August and September did ($p = 0.0233$ and $0.0251$, respectively).

A grouping variable, season of birth, was assessed based on births in December, January, or February ('Winter'); March, April, or May ('Spring'); June, July, or August ('Summer'); and September, October, or November ('Autumn'). Season of birth was associated with NNL width (**Figure 5**) with the wider average NNL in Winter births thinning throughout the year to the narrow NNL observed in Autumn births.

***Pregnancy Outcome: Sex, Gestational Age, and Birthweight***

Because of the relationship ($r^2 = 0.2485$, $p < 0.000$) between the length of gestation and the weight of the infant at birth, it is difficult to separate the potential effect of either factor; however a simple linear regression reveals variation in NNL width correlated with gestational age but not birth weight. Gestational age at birth showed a clear association with NNL width (**Figure 6**). There was no clear reduction or increase in NNL width in babies who were small for gestational age. Width of the NNL increases, or in the case of measurements from nearest the EDJ, at least does not decrease, from Preterm and Early Term births to those at Full Term, and then falls markedly in Late Term and Post Term births. There were no sex-based

differences observed either on their own or in combination with other variables. There was no association between NNL width and crown-heel length of neonate.

***Birth process and early neonatal life variables***

Previous studies have linked NNL width to several factors relating to the mode of delivery, reflecting the potential impact of the birth process on fetal homeostasis, or on the recovery of homeostasis in the infant following birth and disruptions to health in very early neonatal life. The sample size for this investigation was limited to the 32 individuals for whom detailed delivery information was available, and results are given in **Table 5**.

***Birth Process***

This study found no association between mode of delivery the and width of the NNL (**Figure 7).** There were three cases each of planned and emergency Caesarean sections recorded in this sample and the means for both planned and elective caesarean NNL widths fall well inside the range for those from non-assisted births. To increase the numbers in the groups, births were grouped into 'Spontaneous' or requiring 'Intervention', which included instrument-assisted and Caesarean section births. There were no differences in NNL width between children in these two groups. There was no association between the duration of the birth process (measured in 1$^{st}$ stage of labor, 2$^{nd}$, or as total duration) and NNL width. Observations of increased fetal heart rate or maternal distress were not associated with increased NNL width.

***Health at Delivery***

Neonatal line width was not associated with variation in APGAR scores at either one or five minutes after birth. Health problems in the first 14 days after birth including jaundice,

pyrexia, and difficulty feeding were not associated with wider NNLs, though there were only two cases of the latter. Babies requiring some form of resuscitation (n = 10) had a thinner NNL line on average but this was not a significant result.

### Variation Within Individuals

A further issue that has not been specifically investigated previously is the extent of variation in NNL width in different teeth, of the same tooth type, from the same individual, which could have serious implications for the interpretation of causal factors in NNL formation. Eight individuals of the 71 included in the study contributed multiple teeth to the study. Variation of NNL width in the same individual was compared to variation across the sample by generating pairwise comparisons of NNL width between all possible pairs of unmatched teeth and comparing variation in NNL width in the unmatched pairs to variation in NNL between pairs of matched teeth – those from the same individual **(Table 6**). The average variation observed in the teeth from the same individuals is lower than that observed in the unmatched teeth, indicating that teeth from the same individual are more likely to have similar NNL widths than any two teeth drawn at random. The average difference between measurements from different teeth from the same individual ranged from 3.40 μm in the cusp, to 2.80 μm in the middle, and 1.91 μm towards the EDJ. However, differences in NNL width between teeth from the same individual are notable, ranging from < 0.01 to 7.85 μm in the cusp (x = 6.17 μm), 0.13 to 5.56 μm in the middle of the line (x = 4.61 μm), and 0.47 to 7.05 μm near the EDJ (x = 3.43 μm). While cases are limited, the variation between NNL widths measured in upper and lower canines from the same individual is not higher than that measured between left and right canines from the same jaw.

## DISCUSSION

This study examined the relationship of variables describing gestation and birth to the width of the NNL in light of conflicting results from previous studies. Associations with season of birth, gestational age, and metabolic disturbances during pregnancy were observed; associations with duration and method of delivery and early neonatal life were not, with the interesting exception of infants who were resuscitated.

The results obtained from the ALSPAC sample are consistent with previous studies that reported that gestational variables contribute more to the thickness of the neonatal line than delivery variables (Zanolli *et al.*, 2011; Talge *et al.*, 2014; Kurek *et al.*, 2015). This study found that NNL width is lowest in late term births of 41 or more weeks gestation. This is consistent with a previous study (Zanolli, et al. 2011), which reported that children both at term and post-term had thinner NNLs than those born preterm (before the beginning of the $37^{th}$ week). Both studies indicate an association between longer gestation, and hence greater maturity at birth, and thinner NNLs. The interdependence of infant birth weight and gestational age is clear in this study and is a well-established phenomenon (Brenner *et al.*, 1976), however neither birth weight, status as small for gestational age, nor an alternate measure of infant size at birth, crown-to-heel length, were associated with changes in NNL width.

This study found a strong association between season of birth and NNL width, with children born in summer and autumn exhibiting thinner NNLs than those born in winter and spring. In contrast, a previous study on children from Poland reported thinner NNLs in children born in Spring and Summer (Kurek *et al.*, 2015). Season of birth is a marker of maternal sunshine exposure during pregnancy and is indicative of maternal Vitamin D status during pregnancy.

In ALSPAC mothers, 25(OH)D measured at approximately 36.3 weeks gestation was strongly related to estimated background UVB during the last trimester of pregnancy (Sayers *et al.*, 2009; Sayers and Tobias, 2009). Furthermore, background UVB during the last trimester of pregnancy fluctuates in a predictable way according to month and season of birth. This results in a direct link between season of birth and maternal Vitamin D status during the last trimester (Sayers et al 2009). Other research has demonstrated that neonatal serum 25(OH)D3 concentrations are related to maternal levels of Vitamin D during the third trimester (El Koumi *et al.*, 2013).

ALSPAC mothers of children born in winter and spring (December to May) experienced lower levels of sunlight during the third trimester of pregnancy than those born between June and November, leading to lower serum total 25-hydroxyVitamin D (Sayers *et al.*, 2009). While finding a strong association between season of birth and NNL width, we find thinner NNLs in babies born in autumn, rather than in spring and summer as found by Kurek *et al* (2015). This could be due to differing levels of seasonal sunlight in the UK and Poland, where the studies were carried out. Vitamin D status during pregnancy may be a common factor underlying a range of parameters that have been linked to NNL thickness in this and previous studies. Observational studies have demonstrated that Vitamin D status during pregnancy is related to numerous aspects of maternal health, including gestational hypertension, preeclampsia and gestational diabetes, and fetal and neonatal development, including birth weight (Day *et al.*, 2015) and neonatal hypocalcaemia (Curtis *et al.*, 2018).

Neonatal hypocalcemia involves a decline in serum calcium and a rise in phosphorus during the first 12-24 hours after birth. Normal adult values are achieved over the subsequent 24 to 48 hours (Kovacs, 2014). A number of authors have discussed the likely association between

NNL width and neonatal hypocalcemia (Norén *et al.*, 1978a; Norén, 1983, 1984; Kurek *et al.*, 2015) but previous research has yielded conflicting results. In one study, infants of diabetic mothers (IDM) had a significantly higher incidence of wider NNL than healthy infants, and this finding was attributed to the higher incidence of neonatal hypocalcemia reported in IDM (Norén *et al.*, 1978a). Subsequent research on deciduous incisors from a relatively homogeneous group of healthy children born full term found no correlation between the width of the neonatal line and the measured values of blood ionized calcium on days 1, 3, and 5 of infant life (Ranggård *et al.*, 1994). Similarly, hypocalcaemia observed in early neonatal life (at least three consecutive days within the first week) induced by repeated blood transfusions is not reported to be associated with a wider NNL (Ranggård *et al.*, 1995). In the current study no association was found between cord calcium and NNL width, but cord calcium values were only available for 13 cases. Further research is needed to understand the possible relationship between the intensity and duration of hypocalcaemia and ameloblast function and enamel development.

Whether Vitamin D or calcium metabolism is involved in determining the width of the neonatal line remains contentious. The observation of increased NNL width in infants born to diabetic mothers by Norén *et al.* (1978) as well as the association with hypertension seen here might also suggest a separate metabolic pathway acting to increase NNL width, one linked to the suite of conditions associated with insulin resistance (Cheung and Li, 2012). There are no cases of diabetes in the sample studied here. The last variable we are able to comment on is maternal obesity. We found that maternal obesity is associated with a narrower NNL, with a clear trend particularly in the cusp, and with large effect size, which does not fit the expected pattern.

The method and duration of the birth process, and the potential maternal and/or neonatal distress caused thereby, do not show a clear association with NNL width in this study. The potential exception here is the *narrower* width of the NNL observed in infants who were resuscitated. Apart from resuscitation, effect sizes were small to negligible for these variables. Postnatal complications such as jaundice, pyrexia, or other early health conditions within the first 14 days of life were not significantly associated with NNL width. This was unsurprising given the strong evidence for the formation of the NNL at the time of birth (Schour, 1936; Weber and Eisenmann, 1971; Skinner and Dupras, 1993). The lack of association does not fully support a model of NNL formation where the width of the line is influenced by disruptions to fetal/infant homeostasis delimited by the experience of birth, contrary to reported findings from some earlier studies (Eli *et al.*, 1989; Canturk *et al.*, 2014; Hurnanen *et al.*, 2017) but in agreement with other studies (Zanolli *et al.*, 2010; Kurek *et al.*, 2015).

Given the strong associations between NNL width and gestational age, it is possible that there is an additional factor to be considered: the *timing* of the formation of the NNL, compared to the schedule of the development of the enamel crown overall. The timing of NNL formation may affect the width of the NNL as the tooth crown has an underlying schedule of enamel formation that varies considerably from cusp to cervix, and from the EDJ to the enamel surface (Birch and Dean, 2009). The distribution of measured values of NNL width across the tooth crown was also examined and found to map closely to an expected pattern of wider measurements of the NNL in the cusp, where enamel secretion rates are higher, and thinner measurements nearer the EDJ where secretion rates are lower (Mahoney, 2015).

Variation in NNL width at different locations along the NNL may reflect the position of the NNL relative to local enamel formation rates. If the NNL formed at a later point in the overall schedule of enamel development, as would be the case in a Late term or Post Term birth, it would be located in an area where enamel forms more quickly. This timing of birth also has implications for the ameloblasts themselves in terms of their ability to buffer disruptions; the argument that ameloblasts at different stages of activity are more or less susceptible to interruption as proposed by Norén (1983) has been previously established (Witzel *et al.*, 2006). The potential interactions between variation in the rate of enamel formation, susceptibility of ameloblasts to disruption, and the position of the neonatal line could provide an alternate, or possibly complimentary, framework for understanding variation in the width of the NNL. Alternately, this clear variation in NNL width with gestational age may indicate that sensitivity to potentially disruptive influences on enamel formation and mineralization varies according to developmental status of the fetus; more mature fetuses may have developed more robust physiologies that are able to buffer the effects of birth on enamel formation more successfully.

A further consideration is the method by which the data on neonatal line width are measured and analyzed, which may interfere with the interpretation of significant causal factors. The current study is based on measurements of NNL width in teeth of a single type, removing a potential source of variation in measured neonatal line thickness. Early studies used an amalgamated sample of multiple tooth types to estimate the width of the NNL and the associations, for instance between NNL width and mode of delivery, that were derived from those data (Eli *et al.*, 1989; Hurnanen *et al.*, 2017) are not evident in this study of a single tooth type.

This study provided an opportunity to measure NNL width in multiple teeth of the same type from the same individual. While the majority of NNL widths from the teeth from the same individual have far less variation than the wider population, there is still variation in NNL width within individuals stretching from the negligible (<0.01 μm) to the considerable (7.85 μm). One factor that may contribute to variation in NNL width in the same individual is variable section obliquity. It is not always possible to produce identical planes of section for analysis in antimere teeth. Given the expectation that NNL width should be the same in the same tooth type in the same individual, the differences we see between *paired* teeth could point to an optical effect resulting even from minimal section obliquity. The buccolingual sections studied here were taken as close as possible to the long axis of the crown running through the cusp tip, dentine and pulp horns. In our view, this is a critical step in obtaining comparable values though some variation in section obliquity might be expected given the difficulty of identifying the true axis of the tooth, particularly in worn teeth.

A major advantage of this study is that it is based on teeth from a large prospective cohort study and does not rely on parental recollection of early life measures. The children are a relatively homogeneous group who were born within a period of 21 months. The teeth represent a well-resourced population that by definition had access to medical care. A disadvantage of the study is that the low numbers of cases with extreme exposures resulted in limited power to detect meaningful differences for some of the comparisons reported. The results of this study concur with other recent research that links variation in NNL width to gestational variables, but specific differences between the results from this study and earlier research demonstrate the need for replication of results in other research settings including more heterogenous and/or less advantaged study groups.

Decades of research have demonstrated that the NNL is a biomarker for birth, a key life history event, reflecting a disturbance to ameloblast secretion resulting from the birth process (e.g. Weber and Eisenmann, 1971), our results have shown that experiences occurring prior to birth appear to have a greater association with NNL width than the birth process. In this specific case the expression of a chronologically resolved accentuated line cannot be attributed solely to events occurring at the time of its formation. The wider implications of this finding for other accentuated lines in enamel need to be explored. The results of this study do not unambiguously support previous assumptions that the width of the neonatal line is a direct measure of the degree of adversity experienced during gestation or the birth process and the amount of time needed to recover. Ameloblasts may respond differently to the changing physiological circumstances surrounding birth as different thresholds of disruption are reached and according to stage of tooth development and the condition of the fetus at birth. Disruptions that retard enamel formation may lead to the formation of a thinner NNL if the rate of enamel growth itself is slowed; ameloblasts at different stages of development may be more or less susceptible to slowing down or even cessation of matrix formation, which might in turn lead to the formation of very thin NNLs in severely disturbed infants. Future research may be able to resolve these questions by addressing the position of the NNL within the enamel crown and along the EDJ, and by careful repeated measures on a non-oblique section of the NNL in regions of different enamel formation rates throughout the tooth. Rates of enamel secretion surrounding NNL formation and enamel ultrastructural changes within the NNL such as crystallite size and orientation as well as shifts in elemental composition across the NNL, may provide additional evidence for how the factors underlying NNL width interact with the process of enamel formation. Understanding the etiological factors behind observed variation in the structure and composition of dental tissues is fundamental for research that seeks to use these parameters as evidence for early lifetime

experiences in living and past individuals, and it is hoped that future research will continue to explore these interactions.

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**Figure Legends**

**Figure 1** Regular structures of dental enamel, nested view. Clockwise from upper right: position of longitudinal section; histological section; magnified area of section showing NNL (black arrow) and accentuated straie of Retzius (white arrow); magnified view of NNL (black arrow) with visible cross striations.

**Figure 2** Illustration of repeated measures (white bars) of the central third of the NNL. Scale bar: 30 μm.

**Figure 3** Distribution of NNL widths in the sample.

**Figure 4**. Maternal 25H[OH]D (Vitamin D) levels by month tested.

**Figure 5**. Season of birth and NNL Width.

**Figure 6.** Gestational age and NNL Width.

**Figure 7.** Delivery Method and NNL Width.

**Tables**

**Table 1**. Measurements of NNL width (averaged in three locations).

**Table 2.** Description of variables examined. *Where cell counts are less than five, they are reported as <5. **Bold indicates significance or large effect size.** ***Italics indicate a medium effect size.***

**Table 3.** NNL Width across maternal variables. *Where cell counts are less than five, they are reported as <5.

**Table 4.** NNL Width across gestational variables. *Where cell counts are less than five, they are reported as <5.

**Table 5.** NNL Width across birth process and early life variables. *Where cell counts are less than five, they are reported as <5.

**Table 6.** Pairwise differences between teeth from the same individual (ten possible pairings in eight individuals), compared to differences from iterative random pairs generated from all individuals.

## Data Sharing and Data Availability Statement

The data that support the findings of this study are available from the Avon Longitudinal Study of Parents and Children (ALSPAC). Restrictions apply to the availability of these data, which were used under license for this study. ALSPAC data and additional data generated from this study are available on application at http://www.bristol.ac.uk/alspac/researchers/our-data/ with the permission of the ALSPAC executive.

**Table 1**. Measurements of NNL width (averaged in three locations)

| Region | Measurements (n) | Min (μm) | Max (μm) | Mean (μm) | sd (μm) |
|---|---|---|---|---|---|
| Cusp | 142 | 2.06 | 30.13 | 12.33 | 5.72 |
| Middle | 213 | 1.80 | 28.45 | 10.21 | 4.46 |
| EDJ | 212 | 1.70 | 28.02 | 7.69 | 3.63 |

**Table 2.** Description of variables examined. *Where cell counts are less than five, they are reported as <5. **Bold indicates significance or large effect size.** *Italics indicated medium effect.*

| NNL Width n = 71 | | | | p value | | | ω2 value (r2) | | | Cohen's D (+ Hedges G) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Variables** | | **Yes / Data Present** | **No** | **cusp** | **middle** | **EDJ** | **cusp** | **middle** | **EDJ** | **cusp** | **middle** | **EDJ** |
| **Maternal Variables** | Maternal Height | 68 | | 0.80 | 0.65 | 0.66 | *-0.14* | -0.04 | -0.05 | **-2.94** | **-3.36** | **-3.85** |
| | Maternal Weight (Pre-Pregnancy) | 67 | | 0.38 | 0.61 | 0.44 | *0.07* | *-0.06* | 0.04 | **-2.30** | **-2.39** | **-2.48** |
| | Maternal BMI (Pre-Pregnancy) | 67 | | 0.20 | 0.28 | 0.33 | 0.00 | 0.00 | -0.01 | **-2.44** | **-3.40** | **-4.95** |
| | *Maternal Obesity (BMI > 30) | <5 | 63 | 0.04 | 0.07 | 0.21 | 0.05 | 0.03 | 0.01 | **3.12** | **3.35** | *0.64* |
| | Parity | 64 | - | 0.89 | 0.88 | 0.57 | -0.04 | -0.04 | -0.02 | **2.74** | **2.89** | **2.88** |
| | Maternal Age | 67 | - | 0.79 | 0.72 | 0.79 | *-0.08* | *-0.06* | *-0.08* | **-1.40** | **-2.14** | **-3.36** |
| | Geriatric Pregnancy (35 y +) | 11 | 56 | 0.12 | 0.21 | 0.11 | 0.02 | 0.01 | 0.02 | *0.51* | 0.42 | 0.47 |
| | History of Hypertension | 10 | 54 | **0.04** | 0.50 | **0.04** | 0.05 | -0.01 | 0.05 | *-0.70* | -0.23 | *-0.70* |
| | Glycosuria | <5 | 63 | 0.70 | 0.51 | 0.26 | -0.01 | -0.01 | 0.01 | **3.09** | **3.30** | **3.46** |
| | *Hypertension in Pregnancy (only) | 7 | <5 | 0.27 | **0.05** | 0.64 | 0.04 | *0.31* | *-0.08* | *-0.75* | **-1.46** | -0.30 |
| | Maternal Vitamin D Levels in Pregnancy (25[O]D nmol/l) | 38 | - | 0.51 | 0.44 | 0.55 | -0.02 | -0.01 | -0.02 | **-2.45** | **-2.46** | **-2.47** |
| | Insufficient Vit D (> 80 nmol/l 25[O]D) | 9 | 29 | 0.73 | 0.67 | 0.17 | -0.02 | -0.02 | 0.02 | 0.13 | 0.16 | *-0.52* |
| | Cord Blood Calcium | 13 | - | 0.85 | 0.58 | 0.84 | *-0.08* | *-0.06* | *-0.08* | **-7.45** | **-7.77** | **-7.96** |
| | Vomiting (at 20-32w) | 8 | 60 | 0.61 | 0.75 | 0.87 | -0.01 | -0.01 | -0.02 | **2.52** | **2.58** | **2.43** |
| | Any Infection (at 20-32w) | 22 | 46 | 0.08 | 0.25 | 0.54 | 0.03 | 0.01 | -0.01 | **3.01** | **3.01** | **3.08** |
| | Any Infection (7 months +) | 12 | 54 | 0.29 | 0.77 | 0.96 | 0.00 | -0.01 | -0.02 | **2.83** | **2.93** | **2.97** |
| | *Injury or Shock (at 20-32w) | <5 | 64 | 0.43 | 0.67 | 0.61 | -0.01 | -0.01 | -0.01 | **2.51** | **2.56** | **2.42** |
| | *Injury or Shock (7 months +) | <5 | 63 | 0.12 | 0.08 | 0.56 | 0.02 | 0.03 | -0.01 | **2.52** | **2.53** | **2.38** |
| | Season of Birth | 71 | - | 0.44 | 0.06 | 0.09 | 0.00 | *0.06* | *0.05* | **2.46** | **2.50** | **2.02** |
| | Length of Gestation (weeks) | 68 | - | 0.28 | **0.02** | 0.12 | 0.00 | *0.06* | 0.02 | **-7.36** | **-9.87** | **-14.45** |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Preterm' (< 37w), 'Early Term' (37 - 38w), 'Full Term' (39 - 40), 'Late term' (41 weeks), 'Post Term' (42+) | 68 | - | 0.12 | **0.01** | 0.18 | *0.05* | **0.15** | 0.04 | **2.38** | **2.38** | **2.11** |
| | Crown - heel height | 55 | **-** | 0.13 | 0.28 | 0.69 | 0.02 | 0.00 | -0.02 | **-6.36** | **-6.53** | **-6.77** |
| | Birthweight (g) | 68 | - | 0.97 | 0.89 | 0.64 | -0.02 | -0.02 | -0.01 | **-8.65** | **-8.65** | **-8.60** |
| | Small for Gestational Age | 9 | 59 | 0.97 | 0.58 | 0.51 | -0.02 | -0.01 | -0.01 | 0.01 | -0.20 | -0.23 |
| | Sex | 34 F, 37 M | - | 0.74 | 0.58 | 0.41 | -0.01 | -0.01 | 0.00 | 0.08 | 0.13 | 0.20 |
| **Birth Process and Early Life Variables** | Delivery Method | 32 | | 0.86 | 0.61 | 0.73 | *-0.11* | -0.05 | -0.07 | **2.24** | **2.37** | **2.00** |
| | Intervention During Birth | 14 | 17 | 0.91 | 0.69 | 0.64 | -0.03 | -0.03 | -0.03 | 0.04 | -0.14 | 0.16 |
| | Total Duration of Labor (hrs) | 28 | **-** | 0.81 | 0.91 | 0.91 | -0.04 | -0.04 | -0.04 | -0.14 | -0.23 | -0.45 |
| | Length of 1st Stage of Labor (hrs) | 27 | **-** | 0.14 | 0.17 | 0.36 | 0.05 | 0.04 | -0.01 | 0.48 | 0.33 | -0.22 |
| | Length of 2nd Stage of Labor (min) | 27 | **-** | 0.69 | 0.83 | 0.85 | -0.03 | -0.04 | -0.04 | **-1.00** | **-1.02** | **-1.07** |
| | *Maternal Distress in Labor | <5 | 28 | 1.00 | 0.80 | 0.68 | -0.03 | -0.03 | -0.03 | 0.00 | 0.13 | 0.22 |
| | Fetal Heart Rate Abnormal | 18 | 10 | 0.84 | 0.32 | 0.59 | -0.04 | 0.00 | -0.03 | 0.08 | 0.39 | 0.21 |
| | APGAR score at 1 min | 32 | - | 0.95 | 0.70 | 0.80 | -0.03 | -0.03 | -0.03 | *0.50* | 0.19 | **-0.96** |
| | APGAR score at 5 min | 32 | - | 0.71 | 0.44 | 0.62 | -0.03 | -0.01 | -0.02 | 0.09 | -0.37 | **-1.92** |
| | Baby was Resuscitated | 10 | 22 | 0.17 | 0.19 | 0.06 | 0.03 | 0.02 | 0.08 | *-0.52* | -0.50 | ***-0.72*** |
| | * Feeding problems (< 14 postnatal days) | <5 | 30 | 0.70 | 0.69 | 0.98 | -0.03 | -0.03 | -0.03 | -0.27 | -0.28 | 0.02 |
| | Pyrexia (< 14 postnatal days) | 12 | 20 | 0.81 | 0.13 | 0.97 | -0.03 | 0.04 | -0.03 | -0.09 | *0.55* | -0.02 |
| | Jaundice Present | 22 | 10 | 0.95 | 0.60 | 0.62 | -0.03 | -0.02 | -0.02 | 0.02 | 0.20 | 0.18 |

**Table 3.** NNL Width across maternal variables. *Where cell counts are less than five, they are reported as <5.

| **NNL Width** | | | | average value (µm) | | |
|---|---|---|---|---|---|---|
| **Variables** | | ***n*** | **No Data** | **cusp** | **middle** | **EDJ** |
| | *sample mean* | *71* | | *12.33* | *10.21* | *7.69* |
| **Maternal Variables** | ***Maternal Height*** | ***68*** | ***3*** | - | - | - |
| | ***Maternal Weight (Pre-Pregnancy)*** | ***67*** | ***4*** | - | - | - |
| | ***Maternal BMI (Pre-Pregnancy)*** | ***67*** | ***5*** | - | - | - |
| | ****Maternal Obesity*** | | ***5*** | | | |
| | BMI < 30 | 63 | | 12.12 | 10.09 | 7.34 |
| | BMI > 30 | < 5 | | 6.82 | 6.61 | 5.75 |
| | ***Parity*** | | ***7*** | | | |
| | First | 27 | | 11.69 | 9.56 | 6.89 |
| | Second | 25 | | 11.18 | 9.80 | 7.01 |
| | Third | 10 | | 14.33 | 11.03 | 6.12 |
| | Fourth | < 5 | | *N/A* | *N/A* | *N/A* |
| | ***Maternal Age*** | | ***4*** | | | |
| | < 35 years | 56 | | 12.23 | 10.14 | 7.50 |
| | > 35 years | 11 | | 10.19 | 8.68 | 6.56 |
| | ****History of Diabetes*** | **0** | **3** | - | - | - |
| | ***History of Hypertension*** | | ***7*** | | | |
| | Yes | 10 | | 14.71 | 10.59 | 8.65 |
| | No | 54 | | 11.21 | 9.69 | 6.90 |

**Table 4. NNL Width across gestational variables. *Where cell counts are less than five, they are reported as <5.**

| NNL Width | | | | average value (μm) | | |
|---|---|---|---|---|---|---|
| **Variables** | | ***n*** | **No Data** | **cusp** | **middle** | **EDJ** |
| | *sample mean* | *71* | | *12.33* | *10.21* | *7.69* |
| **Gestational Variables** | ***Gestational Diabetes** | ***0*** | ***5*** | - | - | - |
| | ***Glycosuria** | | ***5*** | | | |
| | Yes | < 5 | | 10.92 | 8.63 | 5.81 |
| | No | 63 | | 12.05 | 10.10 | 7.48 |
| | **Hypertension in Pregnancy (only)** | | ***3*** | | | |
| | Yes | 7 | | 16.06 | 12.24 | 8.88 |
| | No | 61 | | 11.54 | 6.74 | 8.10 |
| | ***Preeclampsia** | ***0*** | ***5*** | - | - | - |
| | ***Maternal Vitamin D Levels in Pregnancy (25[O]D nmol/l)*** | | ***33*** | | | |
| | Insufficient (< 80 nmol/l ) | 9 | | 12.03 | 10.30 | 7.24 |
| | Sufficient (> 80 nmol/l ) | 29 | | 10.47 | 8.79 | 8.38 |
| | ***Cord Blood Calcium*** | ***13*** | ***58*** | - | - | - |
| | ***Vomiting (at 20-32w)*** | | ***3*** | | | |
| | Yes | 8 | | 12.68 | 9.47 | 7.14 |
| | No | 60 | | 11.71 | 9.94 | 7.30 |
| | ***Any Infection (at 20-32w)*** | | ***3*** | | | |
| | Yes | 22 | | 13.36 | 10.65 | 7.57 |
| | No | 46 | | 11.09 | 9.51 | 7.16 |
| | ***Any Infection (7 months +)*** | | ***5*** | | | |
| | Yes | 12 | | 13.29 | 9.59 | 7.32 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | No | 54 | | 11.59 | 9.96 | 7.28 |
| | ***Injury or Shock (at 20-32w)*** | - | ***3*** | - | - | - |
| | | < 5 | | 13.75 | 10.69 | 7.92 |
| | No | 64 | | 11.70 | 9.83 | 7.25 |
| | ***Injury or Shock (7 months +)*** | - | ***5*** | - | - | - |
| | Yes | < 5 | | 16.28 | 13.66 | 8.13 |
| | No | 63 | | 11.69 | 9.71 | 7.24 |
| | ***Month of Birth*** | | ***4*** | | | |
| | January | < 5 | | 11.32 | 8.82 | 8.14 |
| | February | < 5 | | 13.20 | 13.33 | 7.13 |
| | March | < 5 | | 13.20 | 9.95 | 9.45 |
| | April | 15 | | 14.33 | 12.78 | 9.46 |
| | May | 5 | | 12.42 | 10.41 | 8.14 |
| | June | 13 | | 12.01 | 8.92 | 7.09 |
| | July | 7 | | 10.50 | 8.58 | 6.33 |
| | August | 8 | | 12.22 | 10.39 | 7.45 |
| | September | < 5 | | 9.72 | 9.25 | 7.01 |
| | October | < 5 | | 8.76 | 7.20 | 5.70 |
| | November | < 5 | | 13.07 | 8.33 | 7.05 |
| | December | < 5 | | 11.68 | 9.44 | 6.66 |
| | ***Season of Birth*** | | ***4*** | | | |
| | Winter | 9 | | 12.28 | 12.07 | 7.20 |
| | Spring | 24 | | 13.74 | 11.81 | 9.17 |
| | Sµmmer | 28 | | 11.69 | 9.26 | 7.00 |
| | Autumn | 10 | | 10.77 | 8.27 | 6.63 |
| | ***Gestation*** | | ***3*** | | | |
| | Preterm (< 37 Wks) | < 5 | | 12.20 | 9.95 | 8.16 |
| | Early Term (37-38 Wks) | 13 | | 11.97 | 10.90 | 7.63 |
| | Full Term (39-40 Wks) | 26 | | 13.58 | 11.45 | 7.95 |
| | Late Term (42 Wks) | 18 | | 10.35 | 8.02 | 6.47 |
| | Post Term (42+ Wks) | 8 | | 9.06 | 7.31 | 5.99 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | ***Crown - heel height*** | ***55*** | ***16*** | ***-*** | ***-*** | ***-*** |
| | ***Birthweight (g)*** | ***68*** | ***3*** | ***-*** | ***-*** | ***-*** |
| | ***Small for Gestational Age*** | | ***3*** | | | |
| | Yes | 9 | | 11.76 | 10.55 | 7.81 |
| | No | 59 | | 11.83 | 9.78 | 7.21 |
| | ***Sex*** | | ***3*** | | | |
| | Female | 37 | | 12.10 | 9.92 | 7.32 |
| | Male | 34 | | 12.54 | 10.47 | 8.02 |

**Table 5. NNL Width across birth process and early life variables. *Where cell counts are less than five, they are reported as <5.**

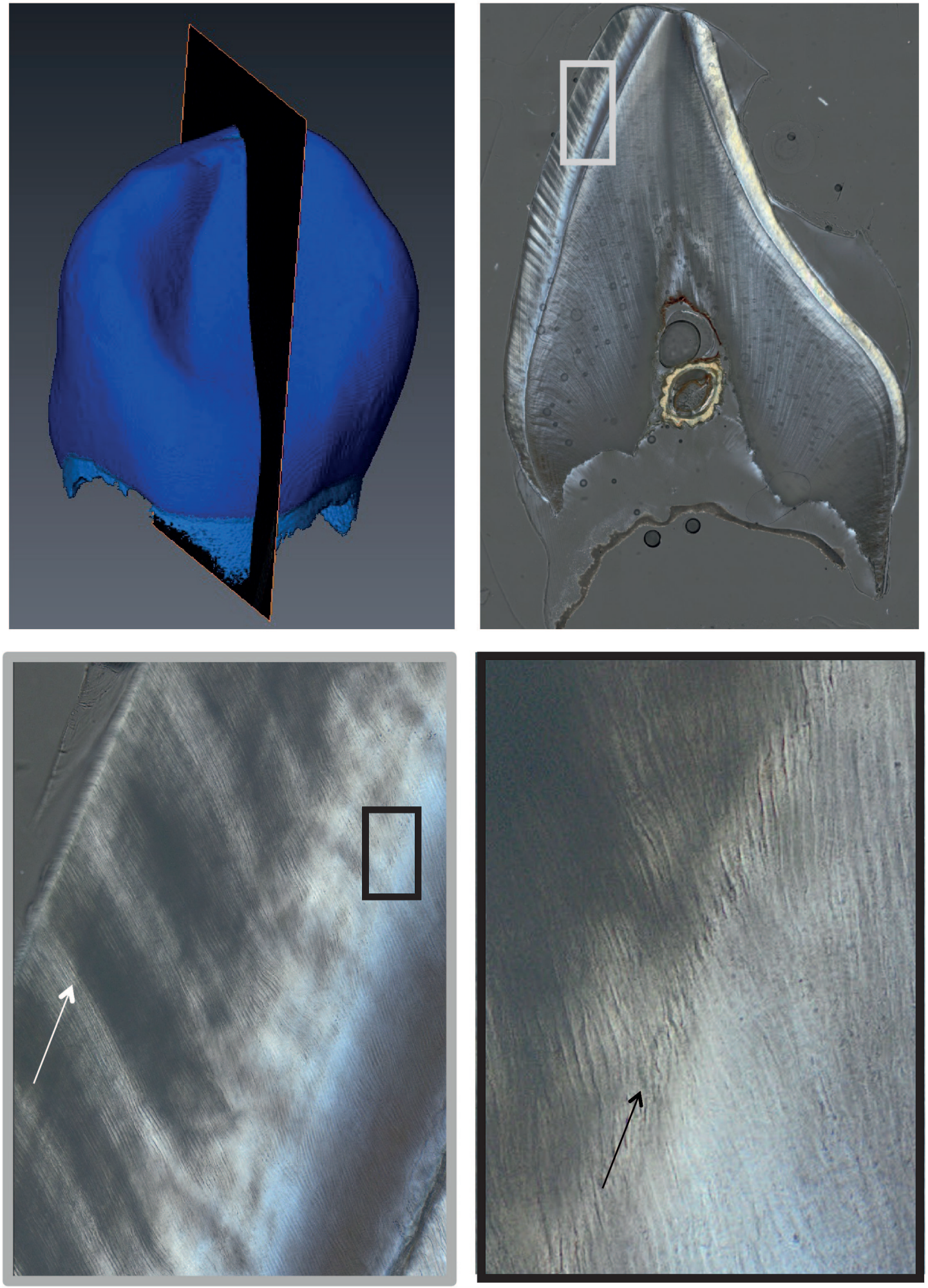
| **NNL Width** | | | | average value (μm) | | |
|---|---|---|---|---|---|---|
| **Variables** | | ***n*** | **No Data** | **cusp** | **middle** | **EDJ** |
| | *sample mean* | *71* | | *12.33* | *10.21* | *7.69* |
| **Birth Process and Early Life Variables** | ***Delivery Method*** | | ***39*** | | | |
| | Spontaneous | 17 | | 10.91 | 9.92 | 7.13 |
| | Assisted Breech | < 5 | | 11.64 | 8.38 | 8.69 |
| | Breech, Extraction | < 5 | | - | - | - |
| | Caesarean Section | 6 | | 12.32 | 9.40 | 7.66 |
| | Forceps | < 5 | | 9.28 | 4.43 | 6.80 |
| | Vacuum Extraction | 5 | | 9.77 | 10.81 | 7.02 |
| | Other | < 5 | | 6.24 | 7.09 | 4.03 |
| | ***Intervention During Birth*** | | ***40*** | | | |
| | Yes | 14 | | 11.10 | 9.40 | 7.52 |
| | No | 17 | 40 | 10.91 | 9.92 | 7.13 |
| | ***Total Duration of Labor (hrs)*** | ***28*** | ***43*** | - | - | - |
| | ***Length of 1st Stage of Labor (hrs)*** | ***27*** | ***44*** | - | - | - |
| | ***Length of 2nd Stage of Labor (min)*** | ***27*** | ***44*** | - | - | - |
| | ****Maternal Distress in Labor*** | | ***39*** | | | |
| | Yes | < 5 | | 10.84 | 10.01 | 7.66 |
| | No | 28 | | 10.84 | 9.54 | 7.14 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | ***Fetal Heart Rate Abnormal*** | | ***43*** | | | |
| | Yes | 18 | | 11.21 | 10.24 | 7.44 |
| | No | 10 | | 10.84 | 8.80 | 6.94 |
| | ***APGAR score at 1 min*** | ***32*** | ***39*** | - | - | - |
| | ***APGAR score at 5 min*** | ***32*** | ***39*** | - | - | - |
| | ***Baby was Resuscitated*** | | ***39*** | | | |
| | Yes | 10 | | 9.19 | 8.40 | 6.09 |
| | No | 22 | | 11.60 | 10.15 | 7.71 |
| | **** Feeding problems (< 14 postnatal days)*** | | ***39*** | | | |
| | Yes | < 5 | | 9.61 | 8.64 | 7.25 |
| | No | 30 | | 10.93 | 9.67 | 7.20 |
| | ***Pyrexia (< 14 postnatal days)*** | | ***39*** | | | |
| | Yes | 12 | | 10.58 | 10.80 | 7.18 |
| | No | 20 | | 11.00 | 8.89 | 7.22 |
| | ***Jaundice Present*** | | ***39*** | | | |
| | Yes | 22 | | 10.88 | 9.83 | 7.34 |
| | No | 10 | | 10.76 | 9.11 | 6.90 |

**Table 6. Pairwise differences between teeth from the same individual (ten possible pairings in eight individuals), compared to differences from iterative random pairs generated from all individuals.**

| Pairwise Differences | **Same Individual (n = 10)** | **All Possible Pairs (n = 2485)** |
|---|---|---|
| | **mean (µm)** | **mean (µm)** |
| **NNL Width at Cusp** | 3.40 | 6.17 |
| **NNL Width at Middle** | 2.80 | 4.61 |
| **NNL Width at EDJ** | 1.91 | 3.43 |

Figure 1. Regular structures of dental enamel, nested view. Clockwise from upper right: position of longitudinal section; histological section; magnified area of section showing NNL (black arrow) and accentuated straie of Retzius (white arrow); magnified view of NNL (black arrow) with visible cross striations.



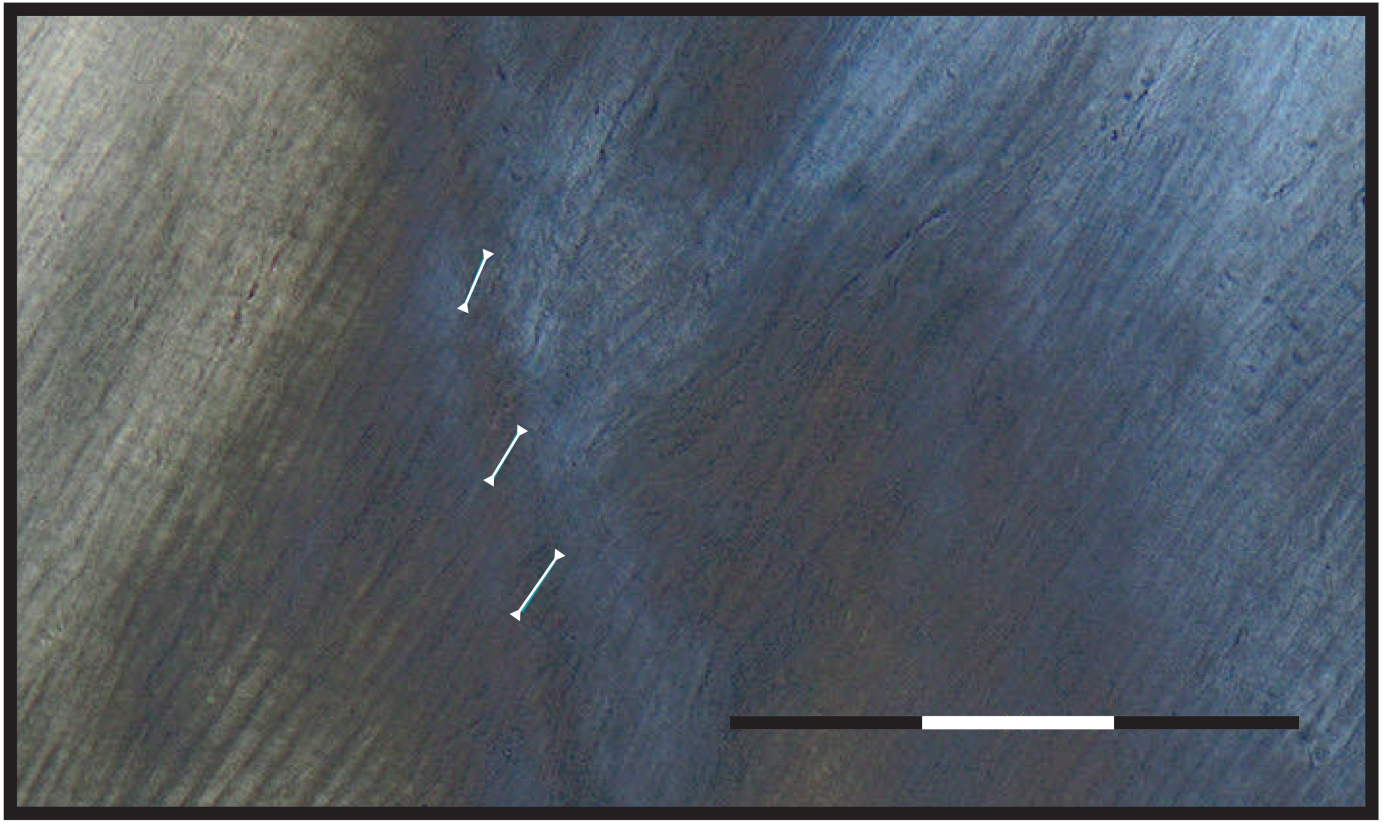


Figure 2 Illustration of repeated measures (white bars) of the central third of the NNL. Scale bar: 30 μm.

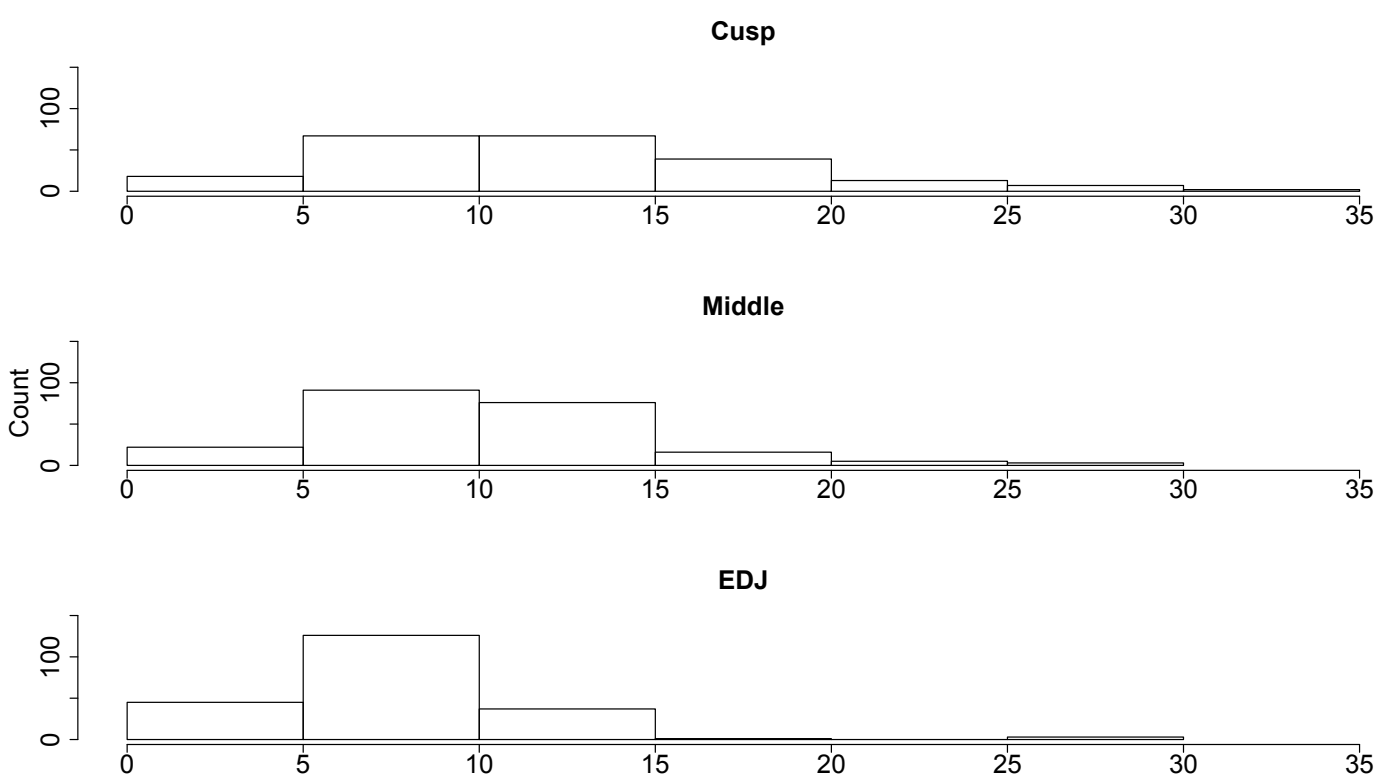
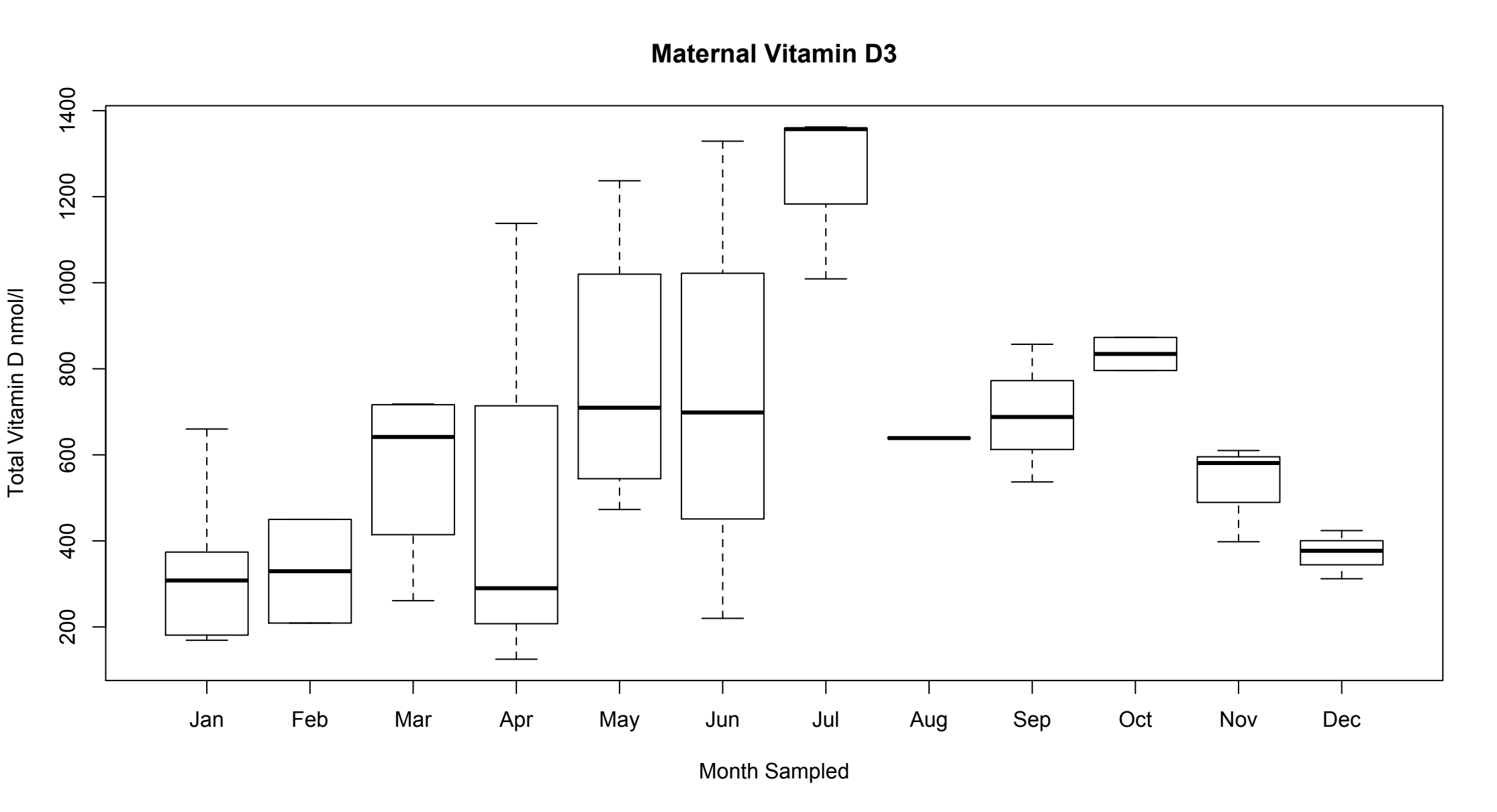



Figure 3. Distributions of NNL width (μm) across the sample.


Maternal Vitamin D3
Total Vitamin D nmol/l
200
400
600
800
1000
1200
1400
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Month Sampled

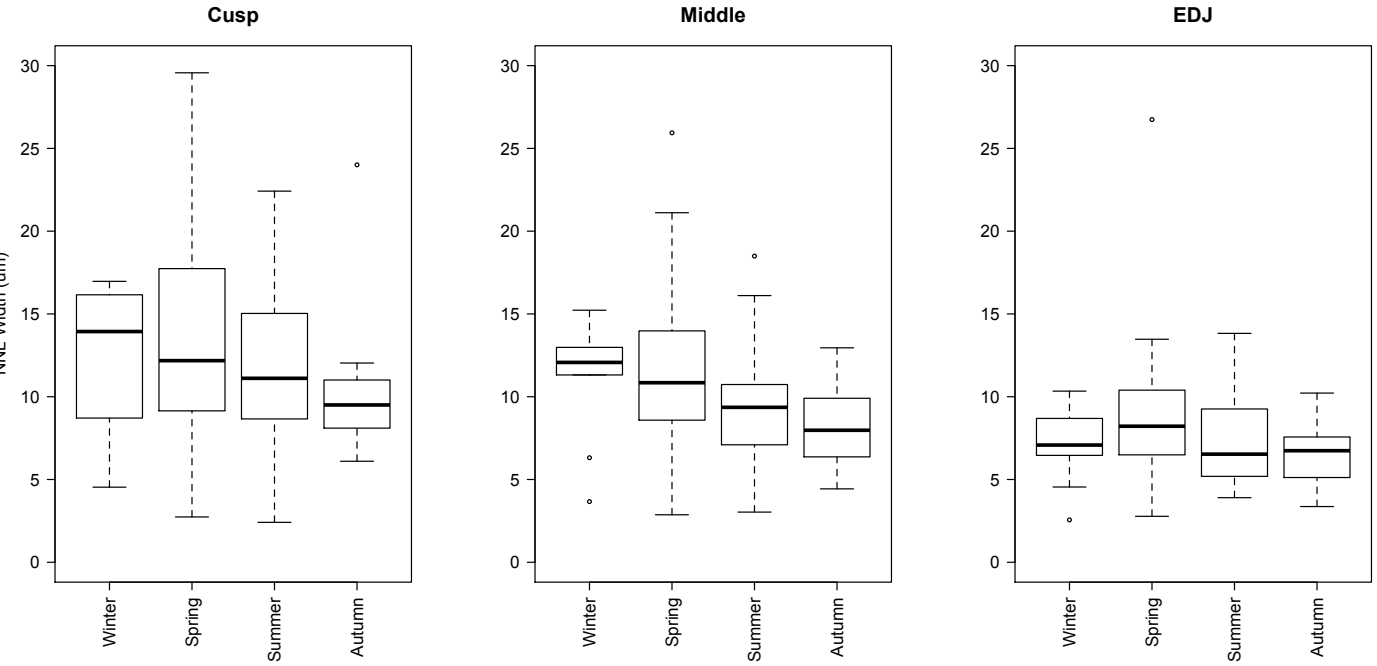



Figure 5. Season of birth and NNL width (μm).

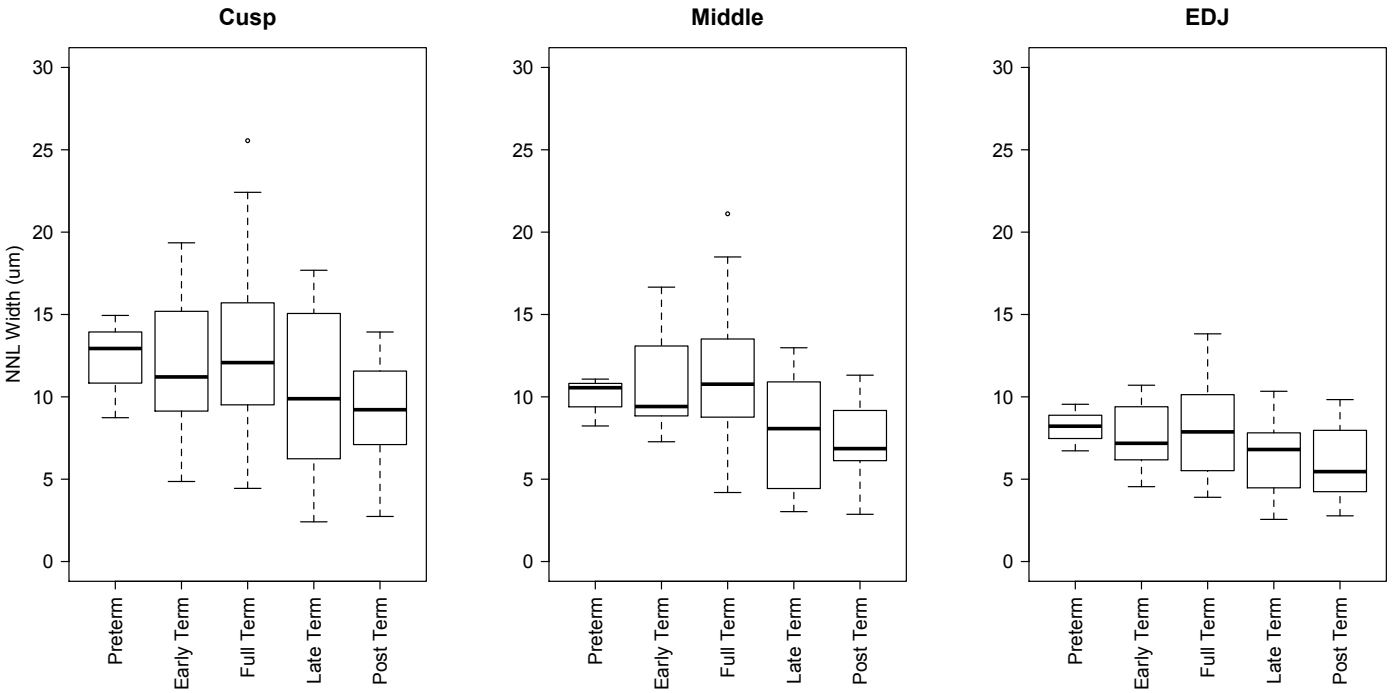



Figure 6. Gestational Age and NNL width (μm).

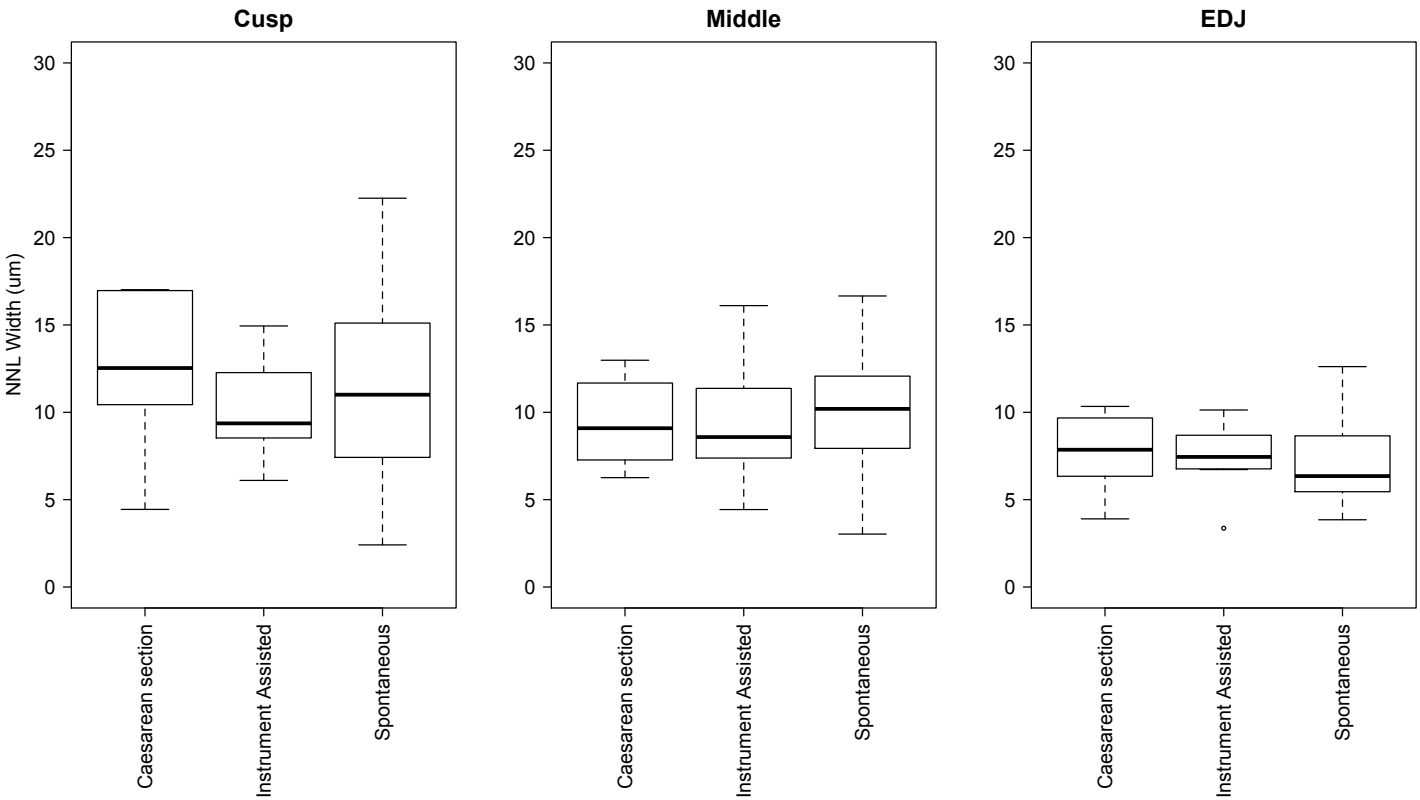



Figure 7. Delivery Method and NNL width (μm).